DETERMINATION OF CORTISOL, CORTISONE, PREDNISOLONE AND PREDNISONE IN BOVINE URINE BY LIQUID CHROMATOGRAPHY–ELECTROSPRAY IONISATION SINGLE QUADRUPOLE MASS SPECTROMETRY

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A quantitative LC-ESI single quadrupole MS method for the determination of cortisol (F), cortisone (E), prednisolone (PL), and prednisone (PN) in bovine urine has been developed and validated. After addingflumethasone as internal standard, the samples were subjected tofiltration, deconjugation, and solid-phase extraction, while the chromatographic separation was achieved using a Restek Ultra II Allure Biphenyl column with isocratic mobile phase. The analytes were detected after negative electrospray ionization using SIM mode. In order to obtain spectra with maximum intensities of at least one of the three characteristic ions, (Mþformate) , (M—H), and [(M—H) - CH_20]~, an individual optimization of MS parameters for each corticosteroid was set up. MS data was acquired in the three-ion selected monitoring mode and the ion ratios between chosen diagnostic ions were used in order to increase the specificity. Calibration graphs were linear and the intra-day and intermediate precision was estimated as RSD values which were less than 17%. For F and E, obtained values indicated negligible absolute matrix effects (10^3% and 98%, respectively). The method was applied to real samples, and basal levels ofFandE were preliminarily evaluated, while PL and PN were not detected.

Keywords bovine urine, cortisol, cortisone, LC-ESI-MS, prednisolone, prednisone

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INTRODUCTION

Natural corticosteroids, as cortisol (F) and cortisone (E), are hormones that are involved in a wide range of physiopathological processes, such as stress response, inflammation, immune function, hydro-electrolyte balance, reproduction, and behavior. F is secreted by the adrenal glands and E, not active hormone, is produced from F by the 11b-hydroxysteroid dehydrogenases (11bHSDs). Both naturally-occurring and synthetic corticosteroids are used in therapeutic medicine for their anti-inflammatory and immunosuppressive actions. Prednisolone (PL), a glucocorticosteroid used as a therapeutic agent in livestock, has also been recently found in cow urine as an endogenous product of corticosteroid metabolism. [1]Prednisone (PN) can be considered an inactive precursor of PL; therefore, it can be linked to the same metabolism.

Effects of the administration of synthetic corticosteroids on urinary F profile were studied in more detailed in humans. [2] On the another hand, only a few studies were undertaken to analyze natural corticosteroids in the urine of bovine treated or untreated with synthetic corticosteroids. [Although the presence of endogenous corticosteroids (F and E) was evaluated in the urine of animals only information about the mode of excretion rates was reported, without providing reference values.[5] Furthermore, PL, that is administered at a low dose (alone or within the protocol containing estrogens and b-agonists), can be metabolized by drug metabolizing enzyme (DMEs) that are mostly abundant in liver. The basic knowledge about cattle DMEs is still superficial and it has not been established yet whether synthetic glucocorticoids interfere with natural corticosteroids' metabolism. For example, in humans, it was con-firmed that PL influences the production of F by inhibiting the hypotha-lamic pituitary adrenal axis[6] and that the F=E ratio can be used as a marker of inhibition of 11bHSD type II produced by a specific drug.[7] On the another hand, dexamethasone (DX), a synthetic corticosteroid frequently used as a therapeutic agent, can interfere with F formation in bovine cell culture.[8] Therefore, F and E could be considered as bio-markers for the activity of therapeutic agents. Moreover, in recent years, several research groups evaluated new strategies for the development of indirect assessment methods to identify animals treated illicitly with anabolic substances (both anabolic hormones and corticosteroids).[4] Therefore, it is reasonable to hypothesize that determination of natural corticosteroids in animals treated with synthetic glucocorticosteroids is of great current interest. In this context evaluation of natural corticosteroid content in cattle might be considered as an important tool that could be used in the screening of glucocorticoid abuse.

The analysis of corticosteroids (either endogenous and=or exogenous) is very complicated, as their metabolism gives a large number of metabolites and because of the low concentrations found in urine. The sample

preparation for screening these compounds in human urine has usually been based on a solid phase extraction, followed by an enzymatic hydrolysis and sometimes another extraction for better purification.[2] Detection and assay methods for corticosteroids have often been based on gas chromato- graphic techniques, occasionally coupled with isotope mass spectrometry.[9] However, the determination of corticosteroids in physiological samples presents some difficulties as most of these compounds are thermally labile and their volatility is low. The derivatization step prior to GC-MS analysis is difficult to be optimized, especially if the aim is to detect a large number of natural and=or synthetic corticosteroids. It is also possible to use immunoenzymatic techniques such as ELISA for systematic detection of corticosteroids, but this technique had problems with interpreting the results, because of the differences in compounds cross-reactivity (e.g., possibility of false positive). [10]Recently methods using liquid chromatography mass spectrometry (LC-MS) have been found to be promising techniques for solving the problem of corticosteroid analysis. ' Especially, liquid chromatography tandem MS (LC-MS=MS) method with electrospray ionization (ESI) in negative mode has proved as sensitive and specific although not always available in routine laboratories. LC-MS determination of endogenous corticosteroids faced some problems and prerequisites related to the chemical structure of the steroids. In fact, urine is particularly rich in different steroidic components, which are not always well separated during the chromatographic run. Unfortunately most fragment ions observed in their mass spectra are common to different components; therefore, a complete specificity is not always possible with co-eluting similar compounds. For these

reasons the HPLC method must be able to separate in a satisfactory way the components of interest.

Thus far, little attention has been paid on simultaneous determination of endogenous corticosteroids in bovine urine by means of single quadru-pole mass detector, due to its lower specificity and sensitivity with respect to MS=MS detection, although some methods have been previously reported for another type of biological matrices. ' Meanwhile, little information is available regarding a reference range about the contents of endogenous corticosteroids in bovine urine. Taking into account all these issues, our study was focused on the exploitation of the potentiality of the single quad-rupole MS detection in order to develop an alternative reliable and useful quantitative method for determination of natural F and E and potentially endogenous PL and PN in bovine urine, by a less expensive procedure with respect to the LC-MS=MS procedure.

EXPERIMENTAL Chemicals and Reagents

F, E, PL, PN, and flumethasone (FL) (as internal standard, IS), meth-anol (LC-MS grade), formic acid (98-100%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced with a Milli-Q system (Millipore, Molsheim, France). Beta-glucuronidase from *Escherichia coli* K 12 (EC 3.2.1.31) in a 50% glycerol solution (pH 6.5) was supplied by Roche Diagnostics GmbH (BoehringerManheim, Germany).

Sample Preparation

Samples of bovine urine were collected from two bovine species: ten bul-locks (age range 12—22 mo) and ten cow (age range 36—42 mo). The samples were received in frozen condition and were kept frozen (—20° C) until analy-sis. A 2-mL aliquot of filtered urine was diluted with 2.5 mL of phosphate buffer (0.2 M, pH = 6) and was incubated with 80 mL b-glucuronidase from *E. coli* at 55°C for 2hr. After cooling down to room temperature, 20 mL of I ppm FL as IS was added and each sample was extracted using Oasis HLB cartridge (3mL, 60 mg, Waters, Milan, Italy) with an extraction vacuum system. The following extraction procedure was optimized: the cartridge was conditioned with 3 mL of methanol followed by 3 mL of water. The sample was deposited on the column and washed first with 3 mL of 10% methanol and then with 1 mL of 2% ammonia in 50% methanol. The elution to recover the corticosteroids of interest was performed with 2 mL of methanol. The eluate was evaporated under a stream of nitrogen and reconstructed in 150 mL of mobile phase.

Instrumentation

The method was developed using a Surveyor Plus LC=MS platform comprised of a Surveyor LC Pump and Surveyor MSQ single quadrupole mass spectrometer with electrospray ionization (ESI) (Thermo-Fisher Scientific, Waltham, MA, USA).

HPLC Conditions

Analytical separations were achieved using a 100 x 2.1 mm Restek Ultra II Allure Biphenyl column with 3 micron particles. Binary isocratic profile was developed using water (A) and methanol (B) both with 0.05% of formic acid at a flow rate of 200 mL=min in ratio 45:55. A sample aliquot (20 mL) was introduced into system by manual loop injection.

Mass Spectrometry

A single quadrupole mass spectrometer was interfaced via an ESI probe operated in negative ion mode and the operating conditions were opti-mized by flow injection analysis (FIA). A full scan and many SIM scans were used for MS parameter optimization. For corticosteroid identification, the ion with the highest S=N ratio as quantifier ion and two confirmation ions were selected for each substance (Table 1). However, analytical work was done by recording the SIM chromatogram corresponding to the quantifier ion and the chromatogram acquired in the three-ions selected monitoring mode. Other MS parameters were optimized as follows: probe temperature: 450°C, needle voltage: 4keV, dwell time 0.1 s, scan range 280-480 Da, and span for SIM events 0.5. Chromatograms were monitored in SIM mode as pseudomolecular ion

species, formic ion adducts, at [M þ 45]~ in the cases of PL and FL, while specific [(M — H) - CH_2O]~ ion was used for E and PN determination. Xcalibur software from Thermo was used for the data processing.

Calibration Curve

A standard stock solution of 1 ppm of corticosteroids was prepared in methanol. Standard spiking solutions at concentrations of 1 ppb were prepared by dilution of the stock standard solution. The appropriate amount of standard spiking solution was added to 2 mL of urine specimens from pooled urine samples with a low content of endogenous corticosteroids to prepare five calibration standards at the concentration of added analytes ranging from 2.5 up to 20 ppb. The urine samples spiked with standards were processed according the same sample preparation procedure described previously. The analysis was performed in triplicate, the endogen-ous contents were calculated by extrapolation from the regression lines and the results were plotted on a calibration curve for each substance. The equations of calibration curves were used to calculate unknown corticosteroids concentration in bovine urine.

TABLE 1 Molecular Mass and Specific Diagnostic Ions (Quantifier Ion in Bold) with ESI Parameters of the Targeted Corticosteroids

Compounds	Mr	Diagnostic Ions	Cone Voltage (eV)
PL	360	329.2	100
		359.2	100
		405.3	60
F	362	331.2	60
		361.2	100
		407.0	60
PN	358	327.2	100
		357.2	60
		403.3	100
FL (IS)	410	343.1	100
		379.3	100
		455.3	60
E	360	311.2	80
		329.2	100
		405.0	60

RESULTS AND DISCUSSION Conditions for HPLC and ESI-MS

The majority of analytical methods which deals with corticosteroids determinations utilizes triple quadrupole mass spectrometers (LC-MS= MS).[14,18,19] The application of HPLC single quadrupole MS technique to the determination of corticosteroids in biological samples has received only limited attention.[1]

According to literature, ESI is suitable for glucocorticoids, which can undergo ionization both in positive and negative modes.[20] Although the positive ionization mode gave a stronger absolute response, the negative ionization was selected due to more specific and more characteristic frag-mentation with less background noise. In order to obtain spectra with maximum intensities of at least one of the three ions characteristic (M þ formate) , (M — H)_ and [(M — H) — CH2O] , an individual optimization of MS parameters, especially cone voltage for each corticosteroid, proved to be necessary (Table 1). The quantifications were preferably made on one ion (quantifier ion) that did not show interferences due to matrix and expressed the highest S=N ratio. For F and PL, it was feasible to use the adduct ion (Mþ formate) for quantification, while for PN and E the conditions were adjusted to have as the most intense signal the [(M — H) — CF^O]" ion. When additional confidence is mandatory for analyte identifi-cation, further measures such as the inclusion of multiple ion confirmation criteria have been required. In our experiments from the same run, it was possible to acquire MS data in the three-ion selected monitoring mode and the ion ratios between chosen diagnostic ions were used for confirmation purposes. Representative SIM signal profiles of studied corticosteroids and chromatograms reporting response of the three selected ions are shown in Figure 1 and mass spectra with diagnostic ions of E, F, and FL in Figure 2. As it appears in Figure 2, the ratios between the diagnostic ions were very simi-lar in both standard mixtures and real urine samples. The ion ratios were within the maximum permitted tolerances according to Commission Decision 2002=657=EC.[ ] This type of data acquisition and the fact that each SIM analysis was carried out at low mass resolution (0.5 amu peak width) further increased method selectivity. To our knowledge, this is the first time that this operation mode was used for qualitative purposes.

When selecting the mobile phase for LC-MS, attention should be paid to the influence of mobile phase on the MS sensitivity. Formic acid, as volatile weak organic acid, generally has been used as mobile phase additive for the ESI positive mode, due to favorable protonation of basic compounds. However, this has not always turned out to occur regularly, especially in case of corticosteroids. On the other hand, formic acid has been very often evaluated for the ability to promote negative ion formation in the electro-spray ion source.[20] In our preliminary experiments, during FIA processing, it was observed that low contribution of [M — H]~ ion, regardless the cone voltage, probe temperature, or needle voltage applied. Therefore, for E and FL, molecular ions were omitted from the final method setting. Contemporary, all corticosteroids investigated gave a prominent pseudomo-lecular peak with formic acid with the highest response at the fixed concen-tration of 0.05% in both organic and water phase. Increasing the concentration of formic acid produced a gradual decrease in absolute abundances. The chromatographic conditions were carefully optimized to obtain the complete separation of the examined analytes. It was of particular impor-tance, bearing in mind the common diagnostic ions for some steroids investigated (PL and E, for example). For this purpose the AllureTM Biphenyl stationary phase proved to be particularly suitable for our steroid separation. Its unique composition of biphenyl groups in sterically favorable position-ing enhances the interactions with steroid fused-ring moieties, resulting in better retention and satisfactory selectivity. [22]As for most HPLC separa-tions, absolute retention times slightly change from run to run, thus relative retention times are used as additional confirmatory help.

As regards the choice of internal standard, FL was chosen after preliminary experiments (data not shown) which demonstrated that it did not interfere within chromatographic profile and that it was absent in tested urine samples obtained from untreated subjects.

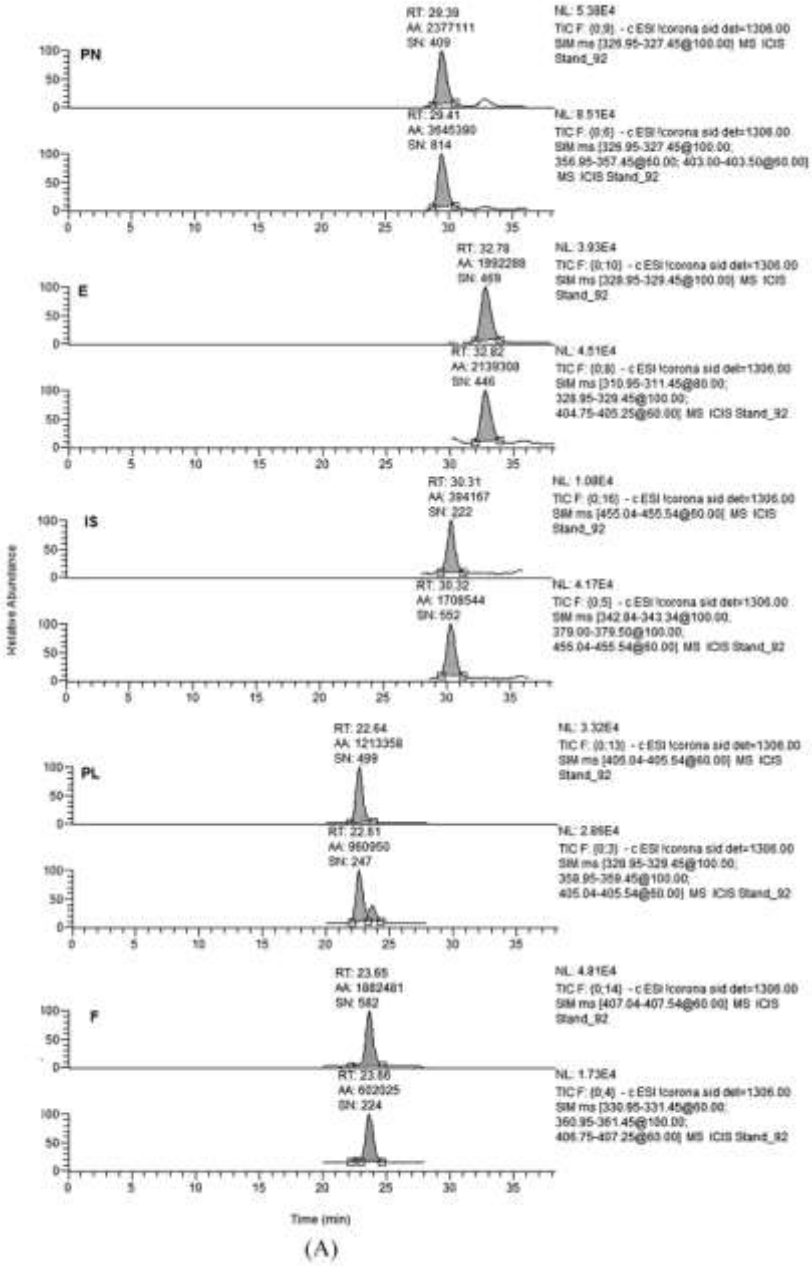
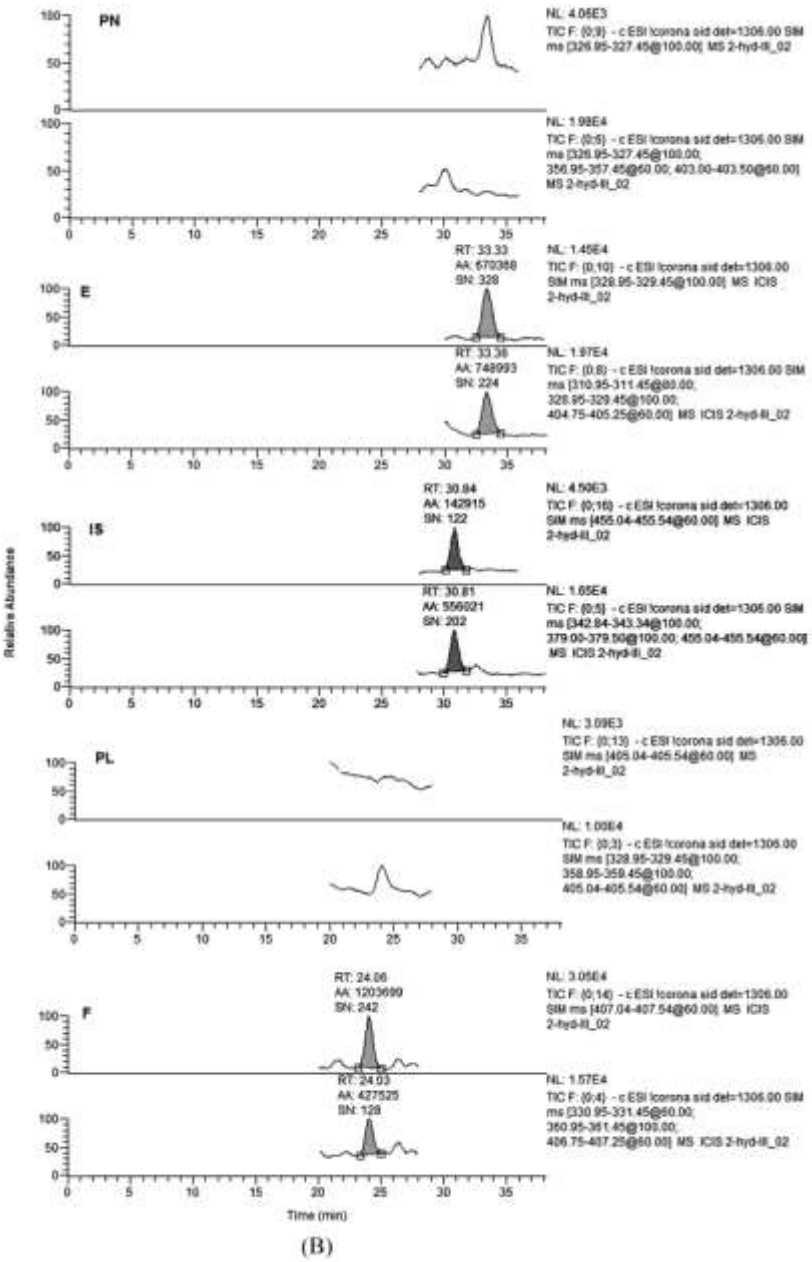


FIGURE 1 LC=MS chromatographic profiles of examined corticosteroids obtained with described conditions in SIM mode for the quantifier ion (upper traces) and as response for the quantifier and the two qualifier ions (lower traces). The total responses of the lower traces can result in lower amounts than upper traces due to the different scanning parameters applied; (A) Standard mixture at 10 ppb; and (B) real urine sample with measured 8 ppb for F and 12 ppb for E.



(B)

FIGURE 1 Continued.

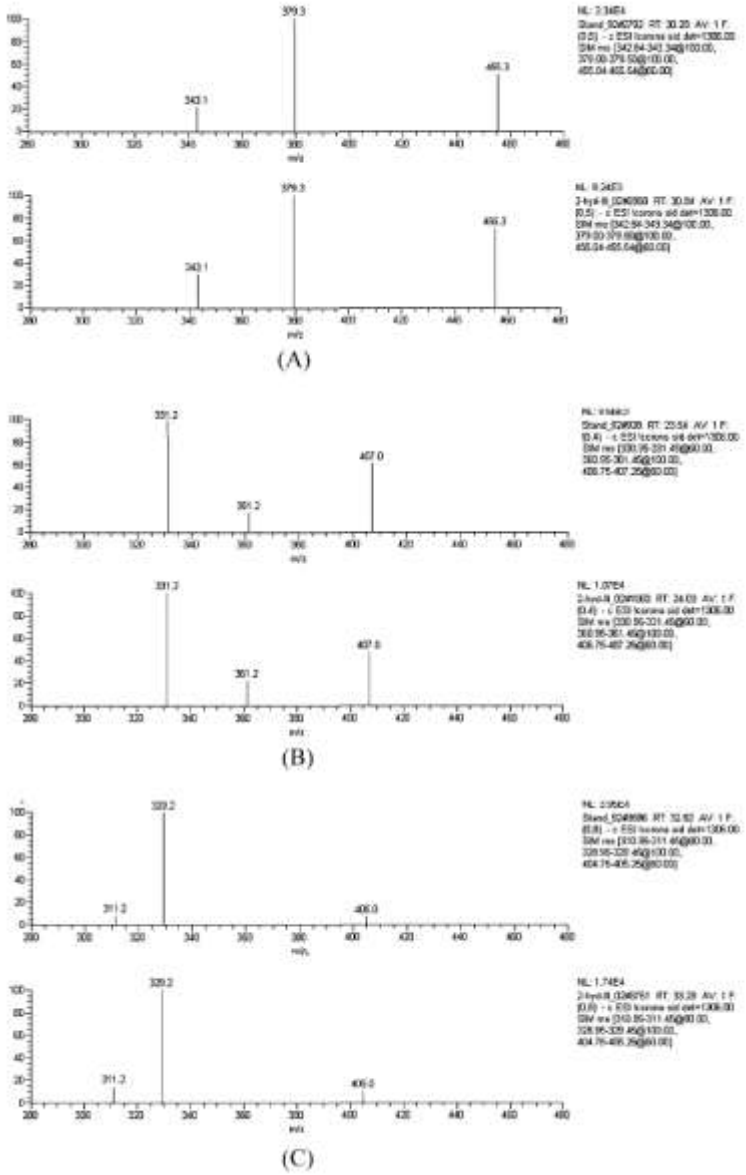


FIGURE 2 MS spectra obtained with three-ion selected monitoring mode, showing the chosen diagnos-tic ions used for confirmation purposes; (A) IS (flumethasone) in standard mix (upper) and in cow urine (36-mo old) (lower); (B) F in standard mix (upper) and in cow urine (36-mo old) (lower); and (C) E in standard mix (upper) and in cow urine (36-mo old) (lower).

Method Validation

Calibration Curve and Linearity

Equal aliquots (in triplicates) of a real pooled urine sample containing the lowest amount of F and E, fortified with five different amounts of corticosteroids (ranging from 2.5—20 ppb) and a constant amount of IS (10 ppb) were subjected to the described procedure. The calibration curve was obtained by reporting Ra versus corticosteroids concentration (endogenous content plus added ppb). By extrapolating from the obtained calibration curve the real pooled urine resulted to contain 2.5 ppb of F and 2.7 of E, while PL and PN appeared undetectable. The coefficients of correlation indicate very good linearity, in the observed concentration range (Table 2). The LOQ was considered the lowest value of each calibration curve that is the real content of F and E and the first spiked quantity of PL and PN (Table 2).

TABLE 2 Validation Results for Targeted Corticosteroids

	Calibration Curve	R^2	LOQ	ME (%)
PL	Ra = 14.05 ppb − 19.61	0.998	2.5	79.6
F	Ra = 92.73 ppb + 30.20	0.997	2.5	103.4
PN	Ra = 31.94 ppb − 63.61	0.988	2.5	108.0
E	Ra = 78.54 ppb + 36.31	0.999	2.7	98.0

Precision

Both instrumental and method precision were studied.

Instrumental System Precision. The same solution at concentration of 10 ppb for each compound was injected 10 times according to the optimal operative conditions to study the repeatability of the instrumental system. Repeatability, expressed as relative standard deviations (RSD), was in range 3.8—5.2% for all corticosteroids analyzed. These results show a good response repeatability of the LC-MS-ESI system. Further, the intermediate precision of the system, again calculated as RSD, was applied to evaluate the variability of the responses between two different days. The values were between 9.1 and 14.7% which was satisfactory considering the detection system used.

Method Precision and Intermediate Precision. Intra-day precision of the assay was determined by replicate analyses of urine samples (n = 6) fortified with 5 ppb of each compound. RSDs were satisfactory for PN (4.9%), E (6.8%), and F (10.5%), while for PL a RSD was 15.6%. The same samples were used for determination of intermediate precision which was obtained from injections in different days. F, E, and PN gave satisfactory RSD values (ranging from 8.4% to 11.2%). Again, PL showed a remarkable variety (RSD = 17%), which can be explained by the fact that, as the most polar among the corticosteroids estimated in this study, had the lowest retention time and interacted with more co-eluting interference substances.

Matrix Effects

The matrix effect (ME) was examined by comparing the mean peak areas of the analytes and the IS between two different series. First series was urine samples (containing 2.5 and 2.7 of F and E, respectively) spiked after the extraction with 2.5 ppb of PL and PN while second series was refer-ence standards (2.5 ppb of F, PL, and PN and 2.7 of E) always followed with 10 ppb for the IS. The ME was defined as following: ME% = 100 x batch 1=batch 2 and it would indicate the possibility of ionization suppression or enhancement for analytes and IS, and an endogenous matrix effect is implied if the ratio was less than 85% or more than 115%, [23]Internal standard FL showed satisfactory value (105.6%) which was an important datum considering that the assessment of matrix effect and assay reliability is critical when similar rather than stable isotope-labeled analytes are utilized as internal standards. The results for examined compounds are presented in Table 2. Ion suppression was observed for PL which was obviously affected by the presence of electrolytes and ionizable co-eluting species. Nevertheless, as other validation data, such as sensitivity and linearity, were acceptable and indicate good performance of the method developed, this was not considered as a drawback for PL accurate determination.

On the contrary, for two natural corticosteroids (F and E), obtained values indicated

negligible absolute matrix effects. This was probably accomplished by introducing washing with basic water=methanol mixture before elution of sample during SPE procedure. This step minimized specific matrix effects of residual compounds and it had been proved as prerogative optimal strategy in endogenous corticosteroids determination. Furthermore, as concerns chromatographic conditions, establishing a prolonged separation run had brought benefit in the signal-to-noise ratio, minimized ion suppression, and improved assay sensitivity.

Application to Real Bovine Urine Samples

The present analytical protocol has been routinely applied in our labora-tory for two types of bovine urine samples. The samples were analyzed and found not containing any other of the monitored steroids than E and F (Table 3). To the best of our knowledge, there are no direct data regarding the basal concentration of these steroids in bovine urine in literature, and this study was undertaken to set up a simple method suitable to obtain infor-mation about their content in different bovine samples. In fact, the sensitivity of the assay was adequate for the determination of these corticosteroids.

The method was applied to some urine samples from subjects with different age and gender. From the preliminary results obtained a high varia-bility appears, especially in bullocks; it seems that older subjects exhibit lower physiological value intervals for F. As F has been considered a chemi-cal marker of stress, these results suggest that younger, male animals could be more subjected to this phenomenon. A larger sample number will be needed in order to obtain significant reference data.

TABLE 3 Concentration of Cortisol and Cortisone in Different Type of Bovine Urine (ppb, Range, MeaniSD)

	Cow ($n=10$, 36–42-mo old)		Bullock ($n=10$, 12–22-mo old)	
	Range	Mean ± SD	Range	Mean ± SD
F	2.8–5.0	3.6 ± 0.84	2.5–12.9	6.2 ± 3.55
E	2.7–20.6	7.6 ± 5.37	3.7–10.4	7.2 ± 2.32

CONCLUSIONS

By exploiting the optimal performances of the available single quadru-pole MS apparatus, a reliable quantification of F, E, PL, and PN was set up with a procedure less expensive than the recently reported ones. Using the proposed method, it would be possible to perform a preliminary monitoring of bovine urine samples, in the view of clinical and eventual inspection purposes. In fact, the method could find application as a non-invasive analytical test for the detection of endogenous corticosteroids, which could be considered possible biomarkers of illegal treatment.

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